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Differences in methylation patterns of the α-fetoprotein and albumin genes in hepatic and non hepatic developing rat tissues

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ABSTRACT

By use of different restriction enzymes sensitive to internal cytosine methylation (HpaII, AvaI, HhaI) we have analysed the methylation patterns of albumin and AFP genes in tissues and cell lines with high (liver, yolk sac, hepatoma cell lines), low (fetal and neonatal kidney) or undetectable (spleen, JF1 fibroblasts) expression of either gene. We show that expression of the AFP gene is associated to the demethylation of a whole region or domain extending from -4 to +3 Kb. Moreover, demethylation of a site located at the upstream limit of this domain appears to be correlated with the commitment of the cell type to synthesize AFP. As concerns the albumin gene, we show that the domain in which demethylation is correlated with active gene transcription in hepatoma cell lines (27) has different borders than in tissue. This difference might be related to the different amounts of mRNA synthesized or to an alteration in gene regulation in tumor cells. Finally, we show that low expression of albumin and AFP genes in fetal and neonatal kidney is not correlated with domain demethylation, suggesting that the regulatory mechanisms of expression of these genes are different in kidney as compared with liver.

INTRODUCTION

Albumin and alphafetoprotein (AFP) are two related serum proteins bearing similar physico-chemical properties. Their concentration in the plasma show considerable variation during development and oncogenesis in mammalian cells (1-3). Rat albumin is synthesized mainly in the liver at all stages of development whereas AFP is only synthesized in fetal and neonatal liver and in the yolk sac (4, 5). AFP is often re-expressed in adult animals bearing hepatomas or teratocarcinomas (1, 2). Although albumin and AFP are considered as markers of hepatic differentiation, recent data revealed low levels of albumin and AFP gene transcripts in non-hepatic tissues such as kidney and pancreas at certain developmental stages (fetal and neonatal) (6,7 Nahon et al., submitted).

Regulation of albumin and AFP production during development and oncogenesis occurs mainly at the transcriptional level (8-13). Change in DNA

methylation was proposed as one of the regulatory mechanisms involved in the control of gene transcription (for review, see 14). However, analysis of the methylation of HpaII sites within rat albumin and AFP transcription units during liver development failed to show a clear correlation between demethylation and expression of these genes (15, 16). On the other hand, some sites of the Xenopus albumin gene were found specifically undermethylated in liver (17). In rat hepatoma cell lines, albumin gene expression has been correlated with the demethylation of an HpaII site located in the first exon (18). These studies were essentially done within the coding regions by use of cDNA clones, and therefore crucial methylated sites located in the 5' non-coding-region might have been missed.

Different lines of evidence demonstrate the importance of the 5' non-coding region of albumin and AFP genes for regulation of their expression : first, this region is required for tissue-specific and developmental regulation of these genes (19-22) ; second, it contains desoxyribonuclease hypersensitive sites (DNAse I H.S. sites) whose presence is associated with the expression of these genes (23, 24). In our laboratory, we have recently shown that the demethylation of specific CCGG sites within the promoter region of the AFP gene is correlated with the gene expression in hepatoma cell lines (25). Similar results were reported for liver from newborn and adult rat (26). On the other hand, it has been shown in several hepatoma cell lines that individual methylation sites of the albumin gene are part of a region or domain in which demethylation is correlated with expression of the gene (27) ; such a domain, rather than single sites, might function as a regulatory element.

To determine whether individual methylation sites of the 5' region of the AFP gene are part of a methylation domain, we have performed an extensive analysis of various methylation sites (HpaII, AvaI, HhaI). These sites are located upstream and within the AFP transcriptional unit and were analysed in AFP-expressing and non-expressing tissues at various developmental stages (developing and adult liver and kidney, yolk sac, adult spleen) and in cell lines (hepatoma and fibroblastic cell lines). In parallel, the degree of methylation of some sites of the methylation domain of the albumin gene (5'-, middle and 3'-limit) was evaluated in the same tissues and cell lines.

Our data shows, for the AFP gene, and confirms, for the albumin gene, the existence of a domain of methylation that is demethylated only in tissues and cell lines where the corresponding genes are highly transcribed.

MATERIAL AND METHODS

Rat tissues and cell lines. Male rats of the Sprague-Dawley strain (Iffa-Credo, St Germain sur l'Arbresle, France) were used to isolate high-molecular weight DNA from the various tissues analyzed. Tissues from fetal and newborn rat were extracted respectively from 19 day-old fetuses and 10 day-old rats.

Hepatoma 7777-C8 is a clonal line derived from the Morris transplantable hepatoma 7777 originally induced in a Buffalo rat (28) ; these cells synthetize AFP but not albumin (15). Hepatoma Faza 2S, kindly provided by Dr. C. Sellem (CGM, CNRS, Gif-sur-Yvette), is a hypertetraploïd line isolated following Sendai virus treatment of Faza 967 (29, 30) : it expresses albumin (31) but not AFP. The JF_1 fibroblast subclone was derived from sarcoma cell line CCL45 (32) ; it does not express either the AFP or the albumin gene.

Preparation of high molecular weight DNA. High-molecular weight DNA was isolated according to the method described by Blin and Stafford (33).

Restriction endonuclease digestions and gel electrophoresis. Aliquots (10 µg) of DNA were digested for one to two hours at 37°C with an excess (10 units/µg of DNA) of HindIII (Amersham) and EcoRI (Boehringer) restriction endonucleases. DNA fragments were then digested for the same time with either MspI (Pharmacia), HpaII, AvaI or HhaI (Boehringer) restriction endonucleases added in excess (10 units/µg of DNA). Electrophoretic separation of the restriction fragments on agarose gels was carried out as previously described (34). Size standards consisted of pBR322 or pBR325 DNA digested with EcoRI, BglI, TaqI and AluI.

Southern transfer and hybridization procedures. Transfer of the separated fragments onto nitrocellulose paper (Schleicher and Schüll) and hybridization with (^{32}P)-nick-translated DNA probes were performed as previously described (34). Cloning and subcloning of the genomic probes used in the methylation analyses were described in previous reports (13, 15, 25).The purified plasmids were labelled by "nick-translation" (35). The specific activity of the resulting recombinant (^{32}P)-DNA was about $2\text{-}4\times10^8$ cpm/µg. Filter autoradiograms were quantitatively scanned with a Vernon densitometer.

RESULTS AND DISCUSSION

The level of methylation of albumin and AFP genes was determined by digestion with the enzymes HpaII, HhaI and AvaI whose cutting is inhibited by methylation of the internal cytosine of their recognition sites, respectively

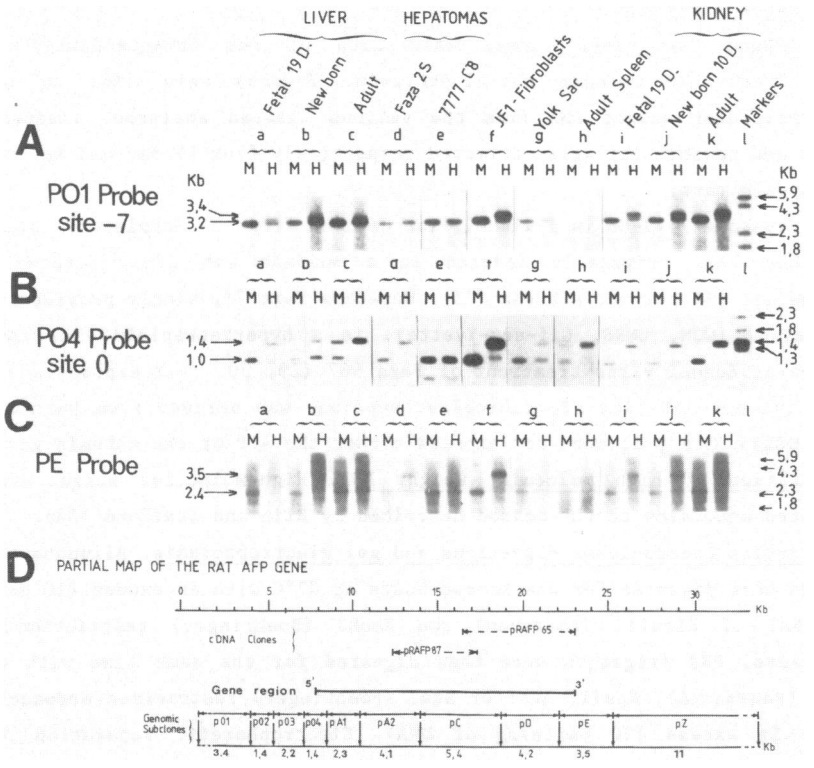

Figure 1 : Methylation of HpaII sites of the AFP gene in various rat tissues.

Genomic DNA (10 µg) extracted from different cell lines and tissues was digested by EcoRI and HindIII then either by HpaII (H) or MspI(M). The resulting fragments were separated on a 1 % agarose slab gel, transferred to nitrocellulose paper and hybridized with PO_1 (A), PO_4 (B) or PE (C) probes which positions on the AFP gene map are indicated in part D.

CCGG, GCGC and $C^{T}_{C}CG^{A}_{G}G$. Partial restriction maps of methylation sites of both genes are represented in fig. 2 for AFP and in fig. 3C for albumin.

Prior to digestion by cytosine-methylation sensitive enzymes, the samples of DNA extracted from various tissues were digested by EcoRI or HindIII. As can be seen on the albumin and AFP genes restriction maps, this double digestion as well as the use of short subclones for probes allowed an analysis of the degree of methylation nearly site by site. When a probed site was fully methylated, a fragment corresponding to the size expected for the EcoRI-HindIII fragment was detected with the probe ; if the probed site was completely demethylated, the EcoRI-HindIII fragment was digested and gave

rise to 2 smaller fragments (or more if it contained more than one site of methylation). For most of the time, a site is only partially methylated and both entire fragments and subfragments resulting from digestion are visible on the autoradiograms (see fig. 1 and 3). The smaller fragments are not shown (fig. 1) since they are not always detected on the autoradiograms. The percentage of methylation of each site was determined from densitometer scans.

We have analysed the degree of methylation of the albumin and AFP genes in different cell lines and tissues where the genes are either highly (more than 1000 copies of mRNA per cell), slightly (100 to 200 copies of mRNA per cell) or not expressed (less than 10 copies of mRNA per cell). The AFP gene is highly expressed in fetal and newborn liver (11, 36), yolk sac (36, 37) and Morris hepatoma 7777-C8 cell line (15). Similarly, the albumin gene is highly transcribed in fetal, newborn and adult liver (11) as well as in hepatoma cell line Faza 2S (10). Low levels of expression of both genes have been detected in fetal and newborn kidney (6, Nahon et al., submitted) whereas transcripts are undetectable in adult kidney, adult spleen and JF1 fibroblasts (15, 25, 36, Nahon et al., submitted).

Methylation of the AFP gene. About 20 methylation sites were analysed in the AFP gene transcription unit. Most of them are located in the coding and non-coding 5' region of the gene (fig. 2). Some of them (two HpaII and one AvaI) are located in the 3' non-coding region (25) and their degrees of methylation were measured by hybridization with PE probe (fig. 1D). Methylation of the central part of the AFP gene could not be measured by use of genomic probes because of the presence of repeated sequences in this region (13) together with a high frequency of methylation sites. However, some of the HpaII sites of this region were analysed in previous reports where cDNA probes were used (15, 16).

Examples of autoradiograms obtained when HpaII digested DNA of various origin was hybridized with AFP genomic subclones are presented in fig. 1. The methylation pattern of the whole 5' region is summarized in fig. 2.

5' end-region. Site 0 is highly methylated in adult liver, hepatoma Faza 2S, JF1 fibroblasts and adult spleen (fig. 1B lanes c, d, f and h), all tissues and cell lines that do not express the AFP gene. Likewise, this site is mostly methylated in kidney at all stages of development (fig. 1B, lanes i, j, k) as we have reported elsewhere (Nahon et al., submitted). In contrast, site 0 is partially or not methylated in fetal and neonatal liver (lanes a, b), in hepatoma 7777-C8 (lane e) and in yolk sac (lane g), all of

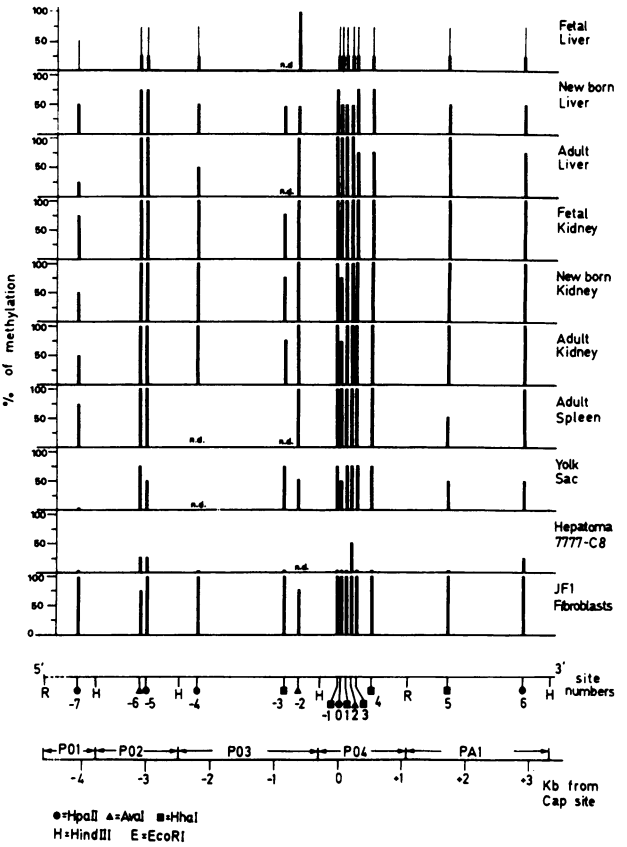

Figure 2 : Summary of the methylation analysis in the 5'-end region of the AFP gene in various rat tissues.

The 5'-end region of the AFP gene is represented as a horizontal line where the sites of methylation analysed are numbered from -7 to +6 with site 0 being the nearest from the cap site. In our previous report where HpaII sites alone were analysed in hepatoma cell lines (24), the sites numbered -3, -2, -1, 0 and +1 correspond respectively, in the present map, to sites -7, -5, -4, 0 and +6. Probe fragments used are indicated below the restriction map. The average percentage of methylation of each site is represented by vertical bars of five possible values : 0, 25, 50, 75 or 100 %. They correspond respectively to scanning values of 0, 0 to 25, 25 to 50, 50 to 75 and 75 to 100 percent of methylation. To show how the cellular heterogeneity can affect the site methylation values, we represented, for fetal liver, both the experimental values (thin bars) and the values expected in the hepatocyte population of the fetal liver (thick bars) when considering that 65 % of the experimental value is contributed by non-parenchymal cells. The latter values were calculated as follows :

$$\text{\% methylation (hepatocytes)} = \left(\text{\% methylation (experimental value)} - \text{\% of non-parenchymal cells}\right) \times \frac{1}{\text{\% of hepatocytes}}$$

which are actively engaged in AFP gene transcription. Thus undermethylation of the site 0 appears correlated with active AFP gene expression.

In all tissues and cell lines analysed, the methylation pattern of the whole 5'-region is similar to that of site 0 (fig. 2) with the exception of site -7. Therefore the region extending from site -6 to site +6 can be considered as a domain of methylation in which demethylation is correlated with AFP gene expression. However, there is an obvious discrepancy in the extent of demethylation of the 5' region between the AFP-expressing tissues and the AFP-expressing hepatoma 7777-C8 (fig. 2). This cannot simply be a consequence of differences in the level of AFP gene expression since yolk sac and hepatoma 7777-C8 synthesize comparable amounts of AFP mRNA. Rather it might be attributed to cellular heterogeneity of tissues compared to cultured cell lines.

Fetal liver (as well as neonatal and adult liver to a lesser extent) is made up of parenchymal cells (hepatocytes, which express albumin and AFP genes) and non-parenchymal cells (which do not express these genes and constitute 60 to 70 % of the fetal liver cells) (38). Comparison of the degree of methylation of adult parenchymal and non-parenchymal cells separated by low speed centrifugation showed that the non-parenchymal cells are hypermethylated at the albumin locus whereas the parenchymal cells are undermethylated (39). It is therefore very likely that during development also, only the hepatocytes are undermethylated at the albumin and AFP gene loci. According to this hypothesis, the percentage of methylation that we have measured could be overestimated, for example in fetal liver, by about 65 %. As shown in fig. 2, taking this value into account lowers the percentage of methylation in fetal hepatocytes by around 25 % (thick bars). A similar correction could be performed on methylation values of the yolk sac, since only its visceral endoderm part (about 50 % of the cells) is engaged in AFP gene expression (40). However, it must be noted that a previous analysis from our laboratory (15), where fetal hepatocytes were isolated by a transient culture step of 4 hours gave results similar to those presented here for fetal liver. In this context, it has been shown that a decline in transcription of liver-specific functions (including albumin) occur within two to four hours of primary culture of mouse hepatocytes (41). It cannot be excluded that changes in DNA methylation accompany the transcriptional alterations that occur during the culture step of isolated hepatocytes.

Site -7, located at the upper limit of the domain of methylation, is mostly demethylated (0 to 50 %) in hepatic tissues and cell lines of hepatic

origin independently of the expression (fig. 1A, lanes a, b, c, d, e). In contrast, it is mostly methylated (50 to 100 %) in cells and tissues of non-hepatic origin (fig. 1A, lanes f, h, i, j, k) except in the AFP-expressing yolk sac where it is completely demethylated (lane g). Demethylation of this site cannot simply be due to correlation with expression since : i) it is demethylated in hepatoma Faza 2S even though this cell line does not express AFP, ii) its methylation decreases from the fetal to the adult stage in parallel with the decrease of AFP gene transcription. Rather, demethylation of site -7 might be correlated with the commitment of a cell type for AFP gene expression since all tissues that actually express or once expressed AFP gene exhibit demethylation of this site. This hypothesis is further supported by the increase in site -7 demethylation that occurs during liver terminal differentiation, concomittantly with an increase in the number of hepatocytes (i.e. AFP committed cells) in this tissue. Moreover, this site is located near a DNAseI H.S. site whose presence is also correlated with the potentiality of a cell type to express AFP in tissues and cell lines (23, 25). Changes in methylation of this site might therefore be involved in modification of interaction with tissue-specific binding factors involved in cell commitment.

3' end region. Three methylation sites (2 HpaII, 1 AvaI) were analysed in this part of the AFP gene. The results obtained for HpaII sites are presented in fig. 1C. Similar results were obtained for the AvaI site (not shown).The presence of repeated sequences in this region (13) has prevented precise measurement of the percentages of methylation. However, the general tendency of the sites to be hyper-, under- or partially methylated could be visually estimated in most cases.

HpaII sites are somewhat hypermethylated in JF1 and Faza 2S cell lines as well as in kidney at all stages of development (Fig. 1C, lanes d, f, i, j, k). In contrast, both sites are somewhat demethylated in 7777-C8 hepatoma cell line (Fig. 1C, lane e).

During development of the liver, from the fetal to the adult stage, the degree of methylation of all three sites decreases, in contrast to that of the sites located in the 5' region (Fig. 1C, lanes a, b, c). This observation is in agreement with results previously reported (15, 16). It is noteworthy that in the partial methylation-patterns of the liver, only two specific bands of high molecular weight are detected even though two HpaII sites are

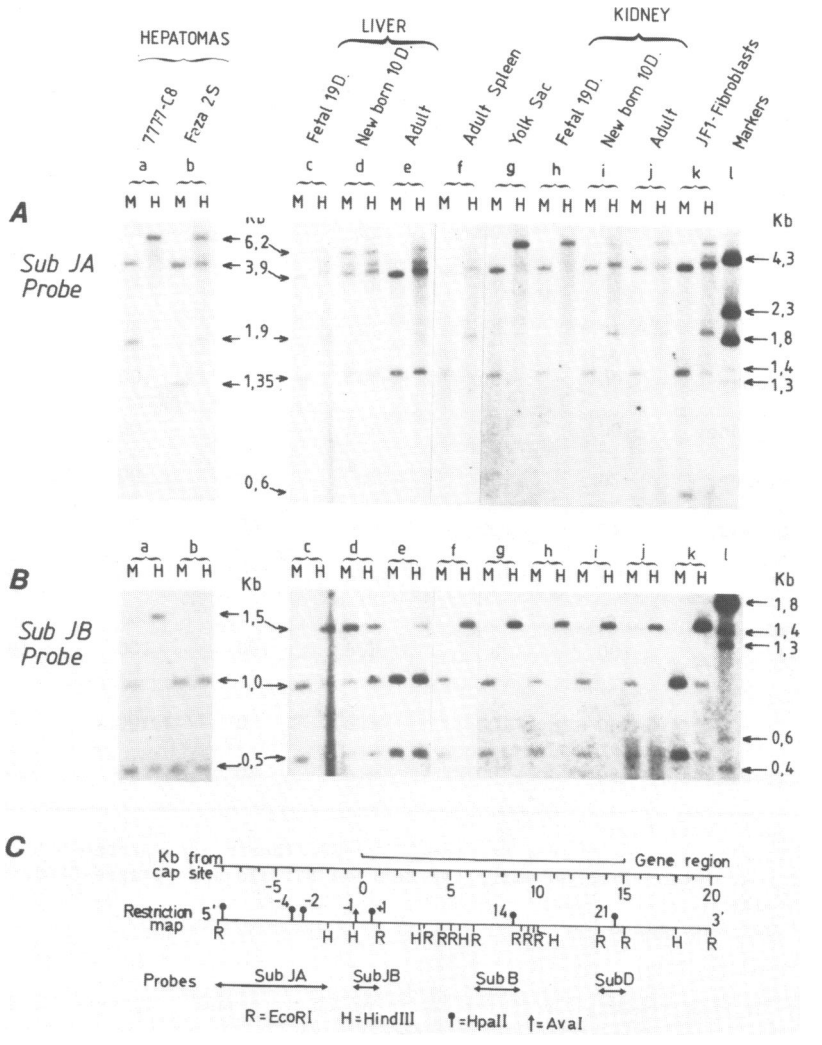

Figure 3 : Methylation of HpaII sites of the albumin gene in various rat tissues.

Genomic DNA (10 µg) extracted from different cell lines and tissues was treated as described in legend of figure 1. After transfer to nitrocellulose paper, the DNA fragments were hybridized with SubJA (A) or SubJB (B) probes. In B, the 1,5 Kb band visible in MspI digestion of lane d is due to incomplete digestion. (C) figures the restriction map of the albumin gene with the position of the probes used. The sites that can be methylated are numbered according to Orlofsky and Chasin (26).

Table 1

Methylation levels upstream and within the albumin gene in various cell lines and tissues

Tissues and cell types	% methylation at site n° :					
	-4	-2	-1	+1	+14	+21
Fetal liver	60	90	50	75	n.d.	n.d.
Newborn liver	75	85	35	45	60	45
Adult liver	50	85	5	10	30	35
Fetal Kidney	100	100	50[a]	95	100	100
Newborn Kidney	100	100	n.d	100	100	100
Adult Kidney	20	100	> 70[a]	100	100	100
Adult Spleen	60	100	n.d	100	100	100
Yolk sac	35	100	n.d	100	100	100
Hepatoma 7777-C8	100	b	n.d	100	100	100
Hepatoma Faza 2S	50	100	n.d	0	100	100
JF1 fibroblasts	50	90	n.d	80	95	100

a : visual estimation
b : this site is absent from hepatoma 7777-C8 albumin gene probably because of restriction site polymorphism between Buffalo and Sprague-Dawley rat strains.
n.d. : not determined.

present in this region (the faint bands of about 6 and 2 Kb must be due to digested repeated sequences of discrete size since they do not correspond to any possible pattern of HpaII digestion of PE). This indicates that the two sites are always simultaneously demethylated or hypermethylated, and thus supports the hypothesis that the methylation percentages of liver sites might be completed in part by a cell population where all sites are fully methylated.

Methylation of the albumin gene. Fig. 3 shows autoradiograms obtained when total DNA of various tissues has been digested by HpaII and hybridized with ^{32}P-labelled albumin probes. The average percentages of methylation deduced from scanning of such autoradiograms are summarized in Table 1.

Previous studies have established a correlation between undermethylation of site +1 and the albumin gene transcription in developing liver (15, 16) and in hepatoma cell lines (18, 27). Our results confirm and extend these conclusions to other tissues and cell lines since site +1 appears fully methylated in all albumin non-expressing tissues and cell lines analysed (fig. 3B, lanes a, f, g, j, k and table 1) and partially or totally demethylated in tissues where the gene is actively transcribed (lanes b to e and table 1). However, the low expression of the albumin gene in fetal and neonatal kidney is not correlated with undermethylation of site +1 (lanes i and j and table 1).

In developing liver, sites 14 and 21, located further along the transcriptional unit, show degrees of undermethylation comparable to those observed for site +1. Contrarily, both sites are fully methylated in hepatoma cell line Faza 2S even though the albumin gene is expressed in this cell line (Table 1). Previous analyses performed on other hepatoma cell lines gave similar results (18, 27). The domain of methylation described by Orlofsky and Chasin (26) might thus have a different 3' border in liver as compared to hepatoma cell lines.

5' non-coding region. In liver during development, the site -1 shows a decrease in methylation similar to that of site +1, i.e. it has the same developmental behavior as the sites located within the coding region (Table 1). Notably, site -1 is the only site that appears undermethylated in fetal kidney as compared to adult kidney (table 1). Since in fetal kidney there is a slight expression of the albumin gene (about 200 molecules/cell), it is possible that demethylation of site -1 is related to the low transcription of this gene.

No obvious correlation is observed between methylation of sites -2 and -4 and the gene expression in any of the tissues or cell lines analysed (Table 1). This result is in good agreement with the results obtained by Ott et al. and Orlofsky and Chasin by analysis of other hepatoma cell lines (18, 27).

CONCLUSION

The present study is concerned with the existence of albumin and AFP gene domain which demethylation would be correlated with gene expression. These domains were found by an extensive restriction enzyme analysis. Since all CpG are not accessible to restriction enzyme, it is possible that some methylated cytosines have escaped our analysis. We have tried to overcome

this limitation by investigating a large number of sites. The homogeneity in the level of methylation detected make us confident that the sites analysed are representative of the whole DNA region considered.

Regarding the AFP gene, extensive analysis of methylation sites in the 5' region lends support to the existence of a methylation domain involved in gene expression, extending from at least -4 kb to + 3 kb. Some of the sites located further than +4 Kb downstream were previously analysed and their methylation degree did not show a clear correlation with expression (15, 16). The end of the domain should therefore be located between +3 and +4 Kb.

As concerns the albumin gene, the existence of a methylation domain was first demonstrated on hepatoma cell lines (27). Our results show that this albumin domain might also exist in developing liver, but with a different 3' limit as compared to hepatoma cell lines. This difference in domain border might be related to the difference in expression. Indeed, a common feature of these hepatoma cell lines is their low production of albumin mRNA as compared to liver (10 to 20 times less : 10, 42). Alternatively, this difference might reflect the alterations of the regulatory mechanisms that probably accompany oncogenesis. These two proposals are not exclusive, and the observation that at least one DNAseI H.S. site upstream from the albumin gene has a different position in the chromatin of hepatoma cell lines as compared to liver (23, 25) is a supportive argument for both hypotheses.

Low expression of albumin and AFP genes in fetal and neonatal kidney do not appear to be correlated with domain demethylation. In a previous report, we showed that this low expression is restricted to the tubular cells in kidney (Nahon *et al.*, submitted). These cells represent about 50 to 70 % of the rat kidney cells at any developmental stage tested here. It is therefore excluded that a consistent gene demethylation in all tubular cells would not be detected. Rather, our results are consistent with the hypothesis that the expression of albumin and AFP genes is regulated differently in kidney as compared to liver. That such might be the case is further supported by the presence of a DNAseI H.S. site specific to kidney cells upstream from the albumin gene (Nahon *et al.*, submitted).

In conclusion, the present study confirms, for the albumin gene, and shows for the AFP gene, the existence of a methylation domain. The regulatory significance of such domains is further supported by the observation that they both contain the sequences necessary for gene expression in transient expression assays (19, 21). Moreover, for the AFP gene, this domain contains

the minimum sequence necessary for tissue-specific and developmental regulation of expression in the liver of trangenic mice (43).

The existence of domains of methylation has been shown or suggested on other systems where numerous sites of methylation were analysed (44-46) or by in vitro methylation experiments (47-48). However, the existence of a methylation domain does not exclude the involvement of individual site demethylation in the control of gene expression.

Changes in DNA methylation can have effects that involve large DNA region. For example, change in methylation has been shown to modify the chromatin structure of the integrated actin gene after transfection in mouse L cells (49) and to facilitate the formation of Z-DNA in CG-rich regions (50). It is possible that such wide range effects would be mediated by domain of methylation.

On the other hand, precise site methylation might be a mechanism for modifying the affinity of a regulatory protein to its binding site. In this context, it has recently been reported that in vitro methylation of a single CG site within a region of the tyrosine amino-transferase gene promoter, target for a transcription factor, prevents the interaction of this factor with its binding site (51). However, not all recognition sequences for binding factors include a CG site. Most probably, the contribution of either sites or domains of methylation in the regulation of a specific gene transcription vary with the gene considered and with its proper regulatory mechanisms.

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+This article is dedicated to the memory of J.M.Sala-Trepat who died while this work was in progress.

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